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Overexpression of *Pto* Activates Defense Responses and Confers Broad Resistance

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The tomato disease resistance (*R*) gene *Pto* specifies race-specific resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato* carrying the *avrPto* gene. *Pto* encodes a serine/threonine protein kinase that is postulated to be activated by a physical interaction with the AvrPto protein. Here, we report that overexpression of *Pto* in tomato activates defense responses in the absence of the Pto–AvrPto interaction. Leaves of three transgenic tomato lines carrying the cauliflower mosaic virus 35S::*Pto* transgene exhibited microscopic cell death, salicylic acid accumulation, and increased expression of pathogenesis-related genes. Cell death in these plants was limited to palisade mesophyll cells and required light for induction. Mesophyll cells of 35S::*Pto* plants showed the accumulation of autofluorescent compounds, callose deposition, and lignification. When inoculated with *P. s. tomato* without *avrPto*, all three 35S::*Pto* lines displayed significant resistance and supported less bacterial growth than did nontransgenic lines. Similarly, the 35S::*Pto* lines also were more resistant to *Xanthomonas campestris* pv *vesicatoria* and *Cladosporium fulvum*. These results demonstrate that defense responses and general resistance can be activated by the overexpression of an *R* gene.

INTRODUCTION

In the long course of plant–pathogen coevolution, plants have developed sophisticated mechanisms to ward off pathogen attack. In addition to preformed physical and chemical barriers, plants have evolved an induced protection mechanism in response to pathogen attack. In many plant–pathogen interaction systems, this induced protection is controlled by the plant disease resistance (*R*) gene (Flor, 1971). *R* genes initiate active defense reactions by recognizing the presence of a corresponding avirulence (*avr*) gene of pathogen origin. In many cases, this gene-for-gene resistance is associated with the hypersensitive response (HR), which is expressed as rapid collapse of the infected tissues (Staskawicz et al., 1995). The HR is often associated with a transient burst of hydrogen peroxide production, cell wall reinforcement through callose deposition and lignification, accumulation of phytoalexins, and activation of defense-related genes (Hammond-Kosack and Jones, 1996). The HR is thought to be an efficient suicide program in plants that halts pathogens at the infection site.

The induction of the HR has been the subject of extensive studies during the past few years. One system in which to study the genetic and biochemical bases of the HR has emerged through the characterization of lesion mimic mutants (reviewed in Dangl et al., 1996). Lesion mimic mutants have been identified in a number of plant species, including maize (Walbot, 1991; Johal et al., 1995), rice (Marchetti et al., 1983), tomato (Langford, 1948), barley (Wolter et al., 1993), and Arabidopsis (Dietrich et al., 1994; Greenberg et al., 1994). These mutants display HR-like or disease lesion–like symptoms in the absence of pathogen infection. Several of these mutants exhibit systemic acquired resistance (SAR), as indicated by enhanced resistance to normally virulent pathogens, the accumulation of salicylic acid (SA), and the constitutive expression of pathogenesis-related (*PR*) genes (Dietrich et al., 1994; Greenberg et al., 1994). The association of defense responses with lesion mimic mutants suggests that genes defined by these lesion mimic mutants may function in disease resistance signal transduction pathways. Because disease resistance is usually linked to the HR, it is conceivable that misregulation or alteration of some components in the disease resistance signal transduction

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pathway may activate cell death in the absence of pathogens. In fact, several maize mutations at the *R* gene locus *rp1* are known to result in a lesion mimic phenotype (Richter et al., 1995; Hu et al., 1996).

The existence of *rp1* lesion mimic mutants strongly supports the possibility that an alteration in *R* gene activity can result in lesion formation and, perhaps, the activation of SAR. However, a lesion mimic phenotype also occurs when several transgenes of plant and bacterial origins are ectopically expressed in plants (Sano et al., 1994; Mittler et al., 1995; Herbers et al., 1996; Abad et al., 1997), indicating that metabolic perturbation may also cause cell death in the plant. Three lesion mimic genes, including maize *Lls1*, barley *Mlo*, and Arabidopsis *Lsd1*, have been cloned (Buschges et al., 1997; Dietrich et al., 1997; Gray et al., 1997). Sequence analyses of these genes, however, provided only limited clues to their biochemical functions and possible roles in normal disease resistance pathways.

In tomato, the disease resistance gene *Pto* in the plant and the *avrPto* gene in the bacterial pathogen *Pseudomonas syringae* pv *tomato* define a gene-for-gene interaction (Ronald et al., 1992; Martin et al., 1993). The *Pto* gene encodes a cytoplasmic serine/threonine protein kinase (Martin et al., 1993; Loh and Martin, 1995). It has been shown previously that a direct interaction of Pto and AvrPto proteins is required for activation of the HR and disease resistance (Scofield et al., 1996; Tang et al., 1996). Here, we report that overexpression of *Pto* under the control of the cauliflower mosaic virus 35S promoter resulted in the formation of microscopic lesions in tomato leaves. Dead cells were localized to palisade mesophyll cells, and the induction of cell death required high-intensity light. The 35S::*Pto* plants exhibited constitutive defense responses, providing race-nonspecific resistance against both bacterial and fungal pathogens.

RESULTS

Overexpression of *Pto* Transcripts in Tomato Transgenic Plants

Seven tomato lines, including Money Maker, Rio Grande–PtoS (PtoS), Rio Grande–PtoR (PtoR), 48-2, 11-12, 13-8, and 11-13, were used in this study. Money Maker and PtoS are two different tomato cultivars that do not carry the *Pto* gene. PtoR is isogenic to PtoS but bears the *Pto* locus introgressed from *Lycopersicon pimpinellifolium* (Martin et al., 1993). 48-2, 11-12, and 13-8 are three independent transgenic lines carrying a *Pto* transgene under the control of the cauliflower mosaic virus 35S promoter. 48-2 is a Money Maker line containing the 35S::*Pto* transgene (Loh et al., 1998). The 35S::*Pto* gene in 11-12 and 13-8 was originally transformed into Money Maker and then backcrossed to PtoS (Martin et al., 1993). 11-12 and 13-8 are derived from the backcrossed progenies and resemble the Rio Grande plants in morphology. 11-13 is a sibling line of 11-12 and was derived from the same progenitor plant; however, it does not contain the 35S::*Pto* transgene. These lines were chosen because the presence or absence of the 35::*Pto* transgene had been previously confirmed by DNA gel blot analysis and bacterial inoculation experiments (data not shown).

We examined the expression of *Pto* transcripts in the transgenic lines by using RNA gel blot analysis. As shown in Figure 1, *Pto* transcripts accumulated to a higher level in all three transgenic lines than they did in PtoR plants. The native *Pto* gene is constitutively expressed at a low level in PtoR plants (Jia et al., 1997) and was not detected by RNA gel blot analysis under the conditions used. The accumulation of *Pto* RNA in 48-2 plants was not observed previously (Loh et al., 1998). The discrepancy might have resulted from insufficient loading of RNA in the previous experiment or improved hybridization conditions in the current RNA gel blot analysis. The weak bands detected in Money Maker, PtoS, and PtoR are derived from a *Pto* family member that is significantly smaller than *Pto* in size.

Pto Overexpression Induces Spontaneous Cell Death

Spontaneous cell death occurred on true leaves of all three 35S::*Pto* transgenic lines when they were grown in the greenhouse (Figure 2A). Dead cells form bleached speckles that can be distinguished from the surrounding, living green cells by using a dissecting microscope. The speckles are scattered on the adaxial surface of mature leaves, barely visible

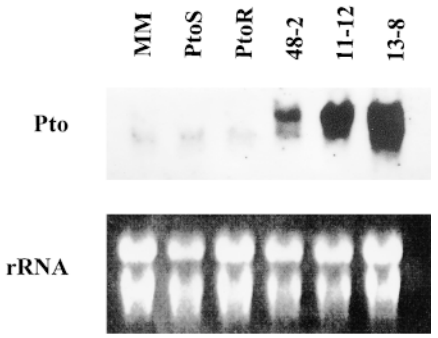

Figure 1. Expression of the *Pto* RNA in Nontransgenic and 35S::*Pto* Transgenic Tomato Leaves.

Total RNA was extracted from fully expanded leaves of 6-week-old plants and analyzed by RNA gel blot analysis. Each lane contained 10 μg of total RNA. The blot was hybridized with the *Pto* cDNA probe (top). Equal loading was verified before blotting by visualizing rRNA in the gel stained with ethidium bromide (bottom). MM, Money Maker.

to the naked eye, and do not have defined margins. These speckles differed from the macroscopic HR in PtoR leaves induced by the avirulent *P. s. tomato* strain T1(*avrPto*) in which the entire inoculated area collapsed. They also differed from the disease lesions caused by *P. s. tomato.* These were dark spots (0.5 to 1 mm in diameter) circled by chlorosis, a symptom caused by the bacterial toxin coronatine. The earliest cell death in plants overexpressing *Pto* was observed on the first true leaf 4 to 5 days after leaf emergence. Once expanded, succeeding true leaves also exhibited cell death. On a single leaflet, cell death started from the tip and extended to the base during leaf development. Cell death was not detected in cotyledons and immature leaves in which cells were not fully differentiated (data not shown). The degree of cell death was constrained because only a portion of cells died within the life span of leaves. The observed cell death did not visibly accelerate the death of the whole leaf. This differs from the propagation type of lesion mimic mutants (Dietrich et al., 1994; Greenberg et al., 1994; Johal et al., 1994). We never observed cell death on leaves of nontransgenic plants before senescence (Figure 2A). The results clearly indicate that *Pto* overexpression causes cell death in the plant.

Hypersensitive cell death is usually correlated with a variety of biochemical reactions, such as the accumulation of autofluorescent compounds, callose deposition, and lignification at and around the lesion site (reviewed in Dixon et al., 1994). These biochemical reactions have been observed in a number of plants with a lesion mimic phenotype (Dietrich et al., 1994; Mittler et al., 1995; Herbers et al., 1996; Abad et al., 1997). To determine whether these responses are associated with cell death in plants overexpressing *Pto*, we assayed the uninoculated leaves of 35S::*Pto* plants for autofluorescence, callose deposition, and lignification. For comparison, we also assayed these reactions at the site of the HR in *P. s. tomato T1*(*avrPto*)–inoculated PtoR leaves.

Analysis of whole-mounted leaves showed strong autofluorescence from leaves of 35S::*Pto* plants (Figure 2C). The autofluorescence was produced by discrete cells. Under the light microscope, the autofluorescence-producing cells were not transparent and thus were different from the nonfluorescent cells, which were clear and transparent (Figure 2B). The distribution of autofluorescence revealed a pattern similar to the scattered yellow speckling observed under the dissecting microscope (Figures 2A and 2C). To correlate the presence of autofluorescence with cell death, we stained the leaves with trypan blue to determine permanent membrane damage (Keogh et al., 1980). The autofluorescent cells were stained blue (data not shown), indicating the death of those cells. The distribution of autofluorescence in 35S::*Pto* plants differed from the HR-associated autofluorescence, which was confluent throughout the inoculation area (data not shown). Autofluorescence was not observed in leaves of the nontransgenic plants (Figure 2C).

Callose deposition at and around the site of the HR is usually part of the complex cell wall–strengthening process that halts pathogen invasion (Stanghellini and Aragati, 1966). To determine whether the scattered cell death in 35S::*Pto* plants is associated with callose deposition, we stained the transgenic *Pto* leaves with aniline blue for the presence of callose. Aniline blue specifically stains callose and produces UV-stimulated fluorescence (Eschrich and Currier, 1964). The fluorescence produced by callose appears bright green and is distinct from the white autofluorescence under the epifluorescence microscope.

Analysis of the whole-mounted leaves stained with aniline blue revealed fluorescent rings in transgenic *Pto* leaves that were not observed without aniline blue treatment. These fluorescent rings likely represent callose deposition along the cell wall (Figure 2D). Cells with callose deposition and cells containing autofluorescent materials were similar in size and shape, and both types of cells were observed within the same focal depth of the microscopic field. These observations suggest that callose and autofluorescent materials are deposited in the same layer of cells. However, the fluorescent rings were usually detected in cells with no autofluorescence, suggesting that callose deposition and the accumulation of autofluorescent materials occur in different cells. We also examined callose deposition at the site of the HR in PtoR leaves. Different from the strong, confluent autofluorescence at the site of HR, fluorescence from callose deposition was barely visible within the dead area. Cells surrounding the dead area did not show callose deposition immediately after the HR but showed strong fluorescence of callose 2 days after the HR developed (data not shown). Callose deposition was not detected in the uninoculated nontransgenic leaves (Figure 2D).

The tomato leaf differentiates into four layers of cells: adaxial epidermal cells, palisade mesophyll cells, spongy mesophyll cells, and abaxial epidermal cells. Although massive yellow speckling was detected on the adaxial side of transgenic leaves, the speckling was not visible from the abaxial side of the leaf (data not shown), indicating that at least the abaxial epidermal cells in the transgenic leaves were not committed to die. To determine whether cell death occurs only in a specific layer of cells, we examined cross-sections of tomato leaves for the presence of autofluorescence. Figure 3A shows that only a small number of palisade mesophyll cells from the 35S::*Pto* plants produced autofluorescence, suggesting that only the palisade cells underwent cell death. This is consistent with the observation that the yellow speckling was detectable only from the adaxial side of the leaf. Dead cells usually accumulate lignin that can be stained red by safranin (Johansen, 1940). Examination of safranin-stained leaf sections with the light microscope indicated that only a small number of palisade mesophyll cells were stained red (Figure 3B). Because lignin is known to produce UV-stimulated autofluorescence (Hammond and Lewis, 1987), we examined the safranin-stained leaf sections for the presence of autofluorescence and found that the autofluorescence was produced by cells staining red (data not shown). These results indicate that the

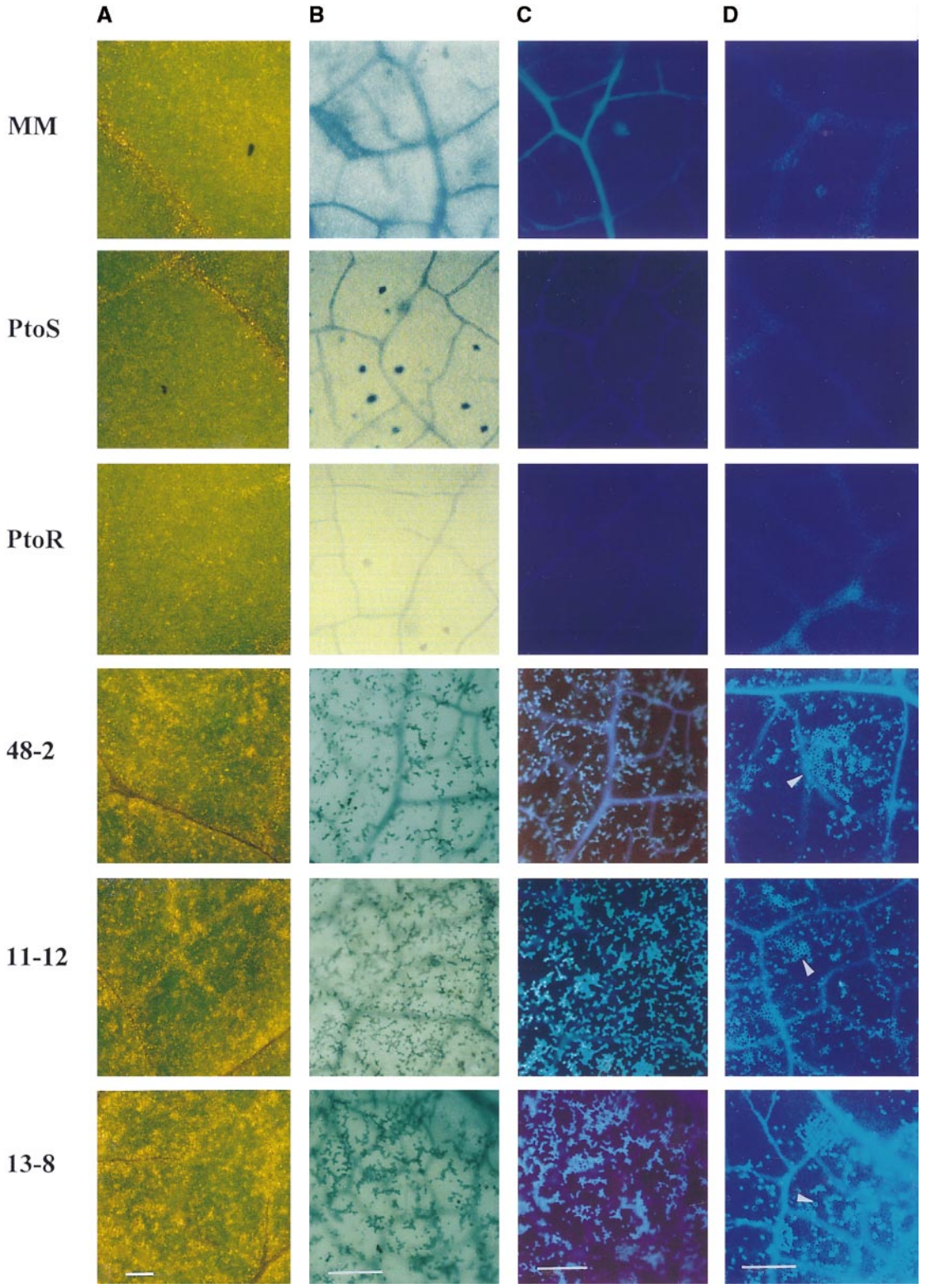

Figure 2. Microscopic Analysis of Whole-Mounted Tomato Leaves.

autofluorescence detected in the whole-mounted leaves was at least in part from lignin.

Cell death in several Arabidopsis and maize lesion mimic mutants is affected by light (Hoisington et al., 1982; Dietrich et al., 1994; Johal et al., 1994). To test whether light affects cell death in transgenic *Pto* plants, we transferred 35S::*Pto* plants grown under natural light (greenhouse) to low light (laboratory). Under low light, the newly emerged leaf took 3 to 4 days longer to undergo cell death than did leaves growing in the greenhouse. In addition, leaves growing under low light produced a smaller number of dead cells than did leaves growing in the greenhouse. To further establish the requirement of light for lesion formation, we covered the young, undifferentiated leaves of greenhouse-grown plants with foil for 7 days. Although this treatment did not significantly block leaf growth, it inhibited cell death in these leaves, indicating that cell death in plants overexpressing *Pto* requires light.

The requirement of light for development of the HR was reported in tobacco and rice infiltrated with incompatible bacterial pathogens (Lozano and Sequeira, 1970; Guo et al., 1993) but not in the *Pto–avrPto* interaction. Therefore, we tested whether the HR induced by the incompatible pathogen also requires light. We injected PtoR leaves with T1, the virulent strain, and T1(*avrPto*), the avirulent strain of *P. s. tomato*, and placed the plants in the dark immediately after inoculation. When an inoculum of 10^7 colony-forming units (cfu) per mL was used, the leaf area injected with T1(*avrPto*) collapsed completely, as a result of the HR, 9 hr after inoculation, whereas the area inoculated with T1 did not exhibit visible changes during the first 24 hr. When the inoculum was decreased to 10^4 cfu/mL, the leaf area injected with T1(*avrPto*) exhibited UV-stimulated autofluorescence from single cells or small cell clusters, indicative of a microscopic HR. In contrast, the leaf area injected with T1 showed disease lesions 3 to 4 days after inoculation. These results indicate that light is not required for the HR mediated by *Pto–avrPto* interaction. The requirement of light for cell death induced by *Pto* overexpression but not for the HR activated by the *Pto–avrPto* interaction suggests that different mechanisms are involved in these processes.

The cell death in plants overexpressing *Pto* also differed from that in the Arabidopsis lesion mimic mutants *lsd1* and *acd1* and the maize mutant *lls1* (Dietrich et al., 1994; Greenberg et al., 1994; Johal et al., 1994). Whereas cell death in the Arabidopsis and maize mutants can be induced by wounding and/or pathogen infection, neither pathogen inoculation nor mechanical wounding augmented further cell death in the transgenic *Pto* leaves (data not shown), suggesting that the molecular mechanisms regulating cell death in plants overexpressing *Pto* are different from those operating during cell death in *lls1*, *lsd1*, and *acd1* mutants.

Pto Overexpression Elevates the Level of SA in Plants

Infection of plants by necrogenic pathogens is known to induce SA accumulation (Ryals et al., 1996). Tomato plants infected by the incompatible strain of *P. s. tomato* also accumulate SA (Oldroyd and Staskawicz, 1998). In lesion mimic mutants, development of spontaneous lesions is often associated with the accumulation of high levels of SA (Dangl et al., 1996; Ryals et al., 1996). The observation of spontaneous cell death in plants overexpressing *Pto* prompted us to examine whether *Pto* overexpression increases the level of SA. We quantitated the SA content in leaves of transgenic *Pto* plants and control plants. As shown in Figure 4, transgenic leaves contained higher levels of both free SA and SA glucoside (SAG). The level of SA was six- to 10-fold higher and the level of SAG was 10- to 20-fold higher in transgenic plants than in nontransgenic plants. The levels of SA and SAG were higher in 13-8 plants than in 11-12 plants, and this coincided with the higher abundance of *Pto* transcripts in 13-8 (Figures 1 and 4). The SA content in 48-2 leaves has not been determined.

Defense Gene Expression in Transgenic *Pto* Plants

Induced *PR* gene expression is common during *R* gene–*avr* gene interactions, including the *Pto–avrPto* interaction

Figure 2. (continued).

Fully expanded leaves from 6-week-old plants were used for microscopic analysis.

(A) Distribution of yellow speckling (dead areas) on the adaxial surfaces of tomato leaves as viewed under a dissecting microscope.

(B) Lactophenol-cleared leaves examined by using a light microscope. Nontransparent cells in transgenic leaves are shown.

(C) UV-stimulated autofluorescence.

(D) UV-stimulated fluorescence from leaves stained with aniline blue. The fluorescent rings indicated by arrowheads represent callose deposition along the cell wall.

The same leaf areas from PtoS, 48-2, 11-12, and 13-8 in **(B)** and **(C)** were photographed and show that identical cells are both nontransparent and autofluorescent. MM, Money Maker. Bars in **(A)** to **(D)** = 200 μm.

(Hammond-Kosack and Jones,1996; Zhou et al., 1997). The induction of *PR* genes is also a hallmark of the plant defense response in many lesion mimic plants (Dietrich et al., 1994; Greenberg et al., 1994; Mittler et al., 1995; Herbers et al., 1996; Abad et al., 1997). The similarities between the plants overexpressing *Pto* and lesion mimic mutants led us to test whether *PR* genes are constitutively induced by *Pto* overexpression. We examined the expression of five *PR* genes: acidic *PR-1* (*PR-1a1*; Tornero et al., 1994), basic *PR-1* (*PR-1b1*; Tornero et al., 1997), acidic chitinase (*Chia*; Danhash et al., 1993), and acidic and basic glucanases (*Glua* and *Glub*; van Kan et al., 1992). Figure 5 shows that the basal expression of all five *PR* genes is higher in the lines overexpressing *Pto* than in the nontransgenic lines, demonstrating that *Pto* overexpression constitutively induces *PR* gene expression. The nontransgenic lines vary in *PR* gene expression, and this may result from the genetic differences in these lines.

The tomato genes *Pti4*, *Pti5*, and *Pti6* encode three transcription factors sharing a similar DNA binding domain that may play an important role in *PR* gene regulation (Zhou et al., 1997). We examined the expression of *Pti4*, *Pti5*, and *Pti6* in the transgenic *Pto* plants. As shown in Figure 5, *Pti4* was expressed constitutively in all tomato lines, independently of the *Pto* gene. However, *Pti5* RNA accumulated to a higher level in plants overexpressing *Pto* than in the nontransgenic plants. The similarities in the expression of *Pti5* and *PR* genes suggest that these genes are regulated by a similar mechanism. Expression of *Pti6* was not reliably detected by the RNA gel blot analysis (probably due to the low abundance of the *Pti6* mRNA) and is not shown.

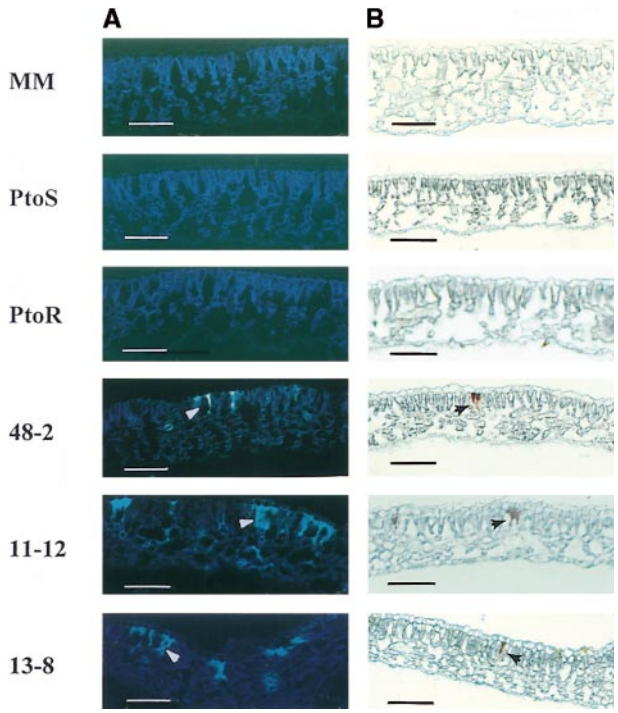

Figure 3. Localization of Dead Cells in Tomato Leaves.

Fully expanded leaves from 6-week-old plants were used for sectioning.

(A) Distribution of autofluorescence in cells of cross-sections of leaf tissue.

(B) Sections stained with safranin showing lignified cells.

Arrowheads indicate cells with autofluorescence and lignification. MM, Money Maker. Bars in **(A)** and **(B)** = 200 μm.

Pto Overexpression Enhances Resistance to a Virulent Strain of *P. s. tomato*

Spontaneous cell death and accumulation of SA and *PR* gene transcripts in plants overexpressing *Pto* indicate constitutive activation of defense system in these plants. Constitutive activation of defense in lesion mimic mutants is known to provide plants with general resistance to a broad spectrum of pathogens (Ryals et al., 1996). To determine whether *Pto* overexpression enhances resistance in a race-nonspecific manner, we inoculated tomato plants with the virulent strain T1 of *P. s. tomato* and examined resistance by comparing disease symptoms and bacterial growth in transgenic and nontransgenic plants. Plants were divided into two groups based on their genetic backgrounds, and resistance was compared among plants (Money Maker versus 48-2; PtoS and PtoR versus 11-12 and 13-8) in each group. When the plants were vacuum-infiltrated with T1 at 10^6 cfu/mL, numerous necrotic lesions developed and eventually became confluent on leaves of both transgenic and nontransgenic plants (data not shown). However, when the inoculum was decreased to 10^4 cfu/mL, leaves of transgenic and nontransgenic plants showed clear differences in disease symptoms.

As shown in Figure 6A, many disease lesions appeared on leaves of the nontransgenic plants (PtoS and PtoR), but only a few lesions developed on leaves of the transgenic plants (11-12 and 48-2). Although many lesions developed on leaves of PtoR plants, the size and density of the lesions were smaller than those on PtoS plants (Figure 6A), suggesting that the *Pto* locus, perhaps the native *Pto* gene, confers a low level of resistance to virulent *P. s. tomato.* The disease symptoms were correlated with the extent of bacterial growth in these leaves. Four days after inoculation, the bacterial number was ~10-fold lower in 48-2 plants than in Money Maker plants (Figure 6C) and 10- to 50-fold lower in 11-12 and 13-8 plants than in PtoR and PtoS plants (Figure 6B). Bacterial numbers in 11-12 and 13-8 lines were also significantly lower (10- to 20-fold) than those in Money Maker and 11-13, two control lines that do not carry the 35S::*Pto* transgene (data not shown). An LSD statistical test showed that the differences in bacterial growth were significant ($P = 0.01$). The race-nonspecific resistance

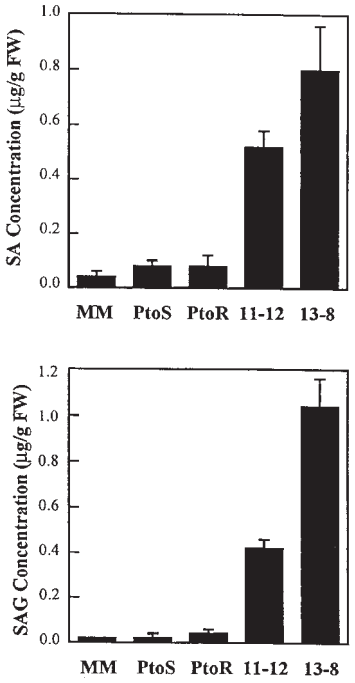

Figure 4. Endogenous Levels of SA and SAG in Nontransgenic and 35S::*Pto* Plants.

SA (top) and SAG (bottom) were extracted from leaves of 6-week-old plants and analyzed by HPLC. The values presented are an average of three replicates. Error bars indicate standard error. FW, fresh weight; MM, Money Maker.

to *P. s. tomato* was much weaker compared with the race-specific resistance triggered by *avrPto*, which inhibited the bacterial pathogen T1(*avrPto*) up to 1000-fold (Figures 6C and 6D).

Pto Overexpression Provides Broad Resistance

To determine whether *Pto* overexpression provides resistance to pathogens in addition to *P. s. tomato*, we infected the tomato plants with *Xanthomonas campestris* pv *vesicatoria* and *Cladosporium fulvum*. *X. c. vesicatoria* is a leaf pathogen that causes bacterial spot disease on tomato, and resistance to race 1 of this pathogen has been detected only in the tomato line Hawaii 7998 (Scott and Jones, 1986). We inoculated two isolates, 75-3 and 90-14, of *X. c. vesicatoria* race 1 bacteria into transgenic and nontransgenic plants. At a concentration of 10^4 cfu/mL, both 75-3 and 90-14 caused severe disease lesions on PtoS and PtoR leaves but few lesions on the transgenic leaves. Figure 7A shows tomato leaves inoculated with 75-3. Leaves inoculated with isolate 90-14 produced similar symptoms. We also measured bacterial growth of 75-3 and 90-14 in leaves of Money Maker and 48-2 and found that the bacterial number was inhibited by ~10-fold in 48-2 plants (Figures 7B and 7C). The difference between the bacterial growth on Money Maker and 48-2 plants was significant in an LSD test ($P = 0.01$).

Overexpression of *Pto* in tomato also increases resistance to the fungal pathogen *C. fulvum*, the causal agent of leaf mold disease, as measured by a β-glucuronidase (GUS) assay of a *C. fulvum* strain expressing the *GUS* gene. Figure

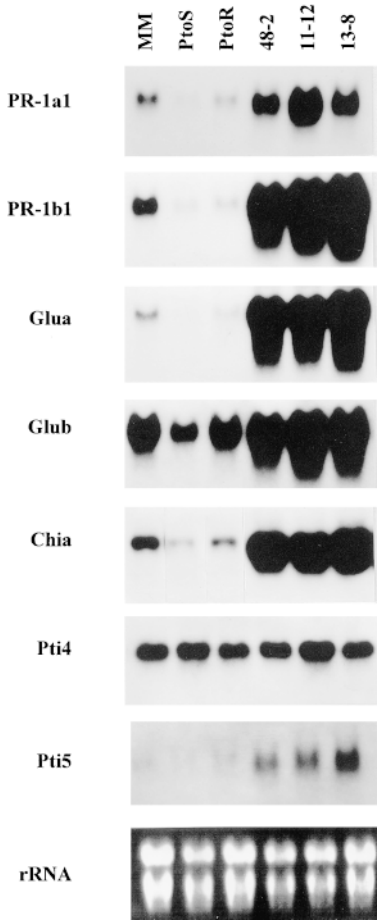

Figure 5. Expression of Defense-Related Genes in Nontransgenic and Transgenic *Pto* Plants.

Total RNA extracted from fully expanded leaves of 6-week-old plants was analyzed by RNA gel blotting. Ten micrograms of total RNA was loaded in each lane. The blots were hybridized with the cDNA probes for *PR-1a1*, *PR-1b1*, *Glua*, *Glub*, *Chia*, *Pti4*, and *Pti5.* Equal loading was verified before blotting by visualizing rRNA in the gel stained with ethidium bromide (bottom). MM, Money Maker.

8 shows the resistance of the transgenic line 11-12. Heavy fungal growth, as shown by the GUS activity in leaf discs, was observed 8 days after inoculation in all tomato lines lacking the 35S::*Pto* transgene. In contrast, the 35S::*Pto* line 11-12 showed little fungal growth. Experiments with transgenic line 13-8 produced similar results (data not shown). The enhanced resistance to *X. c. vesicatoria* and *C. fulvum* in transgenic plants demonstrated that disease resistance conferred by *Pto* overexpression is effective against multiple pathogens.

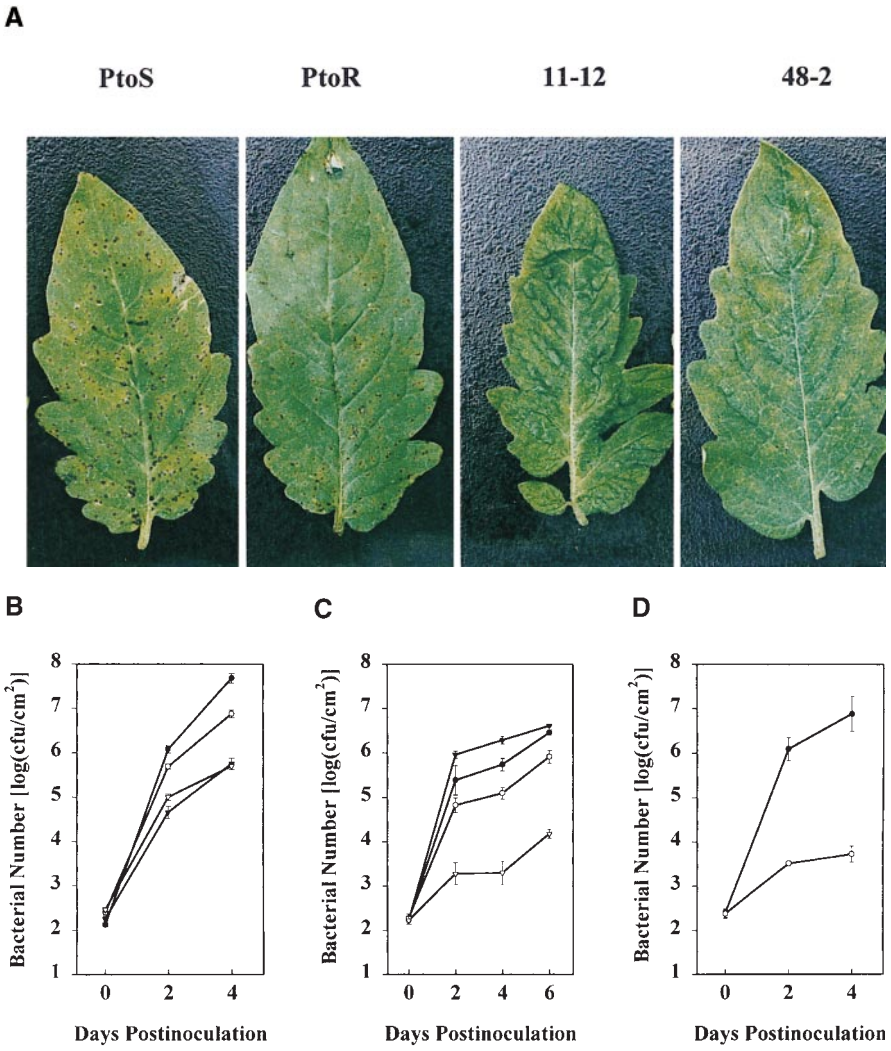

Figure 6. Increased Resistance of 35S::*Pto*–Transformed Plants to *P. s. tomato.*

Six-week-old tomato plants were vacuum-infiltrated with the T1 strain and the T1(*avrPto*) strain of *P. s. tomato* (10^4 cfu/mL, in 10 mM $MgCl_2$ plus 0.04% Silwet).

(A) Disease symptoms caused by *P. s. tomato* T1 infection. Disease symptoms were documented 4 days after inoculation.

(B) Growth of *P. s. tomato* T1 on leaves of PtoS, PtoR, 11-12, and 13-8 plants. Leaf bacteria were measured as described in Methods. Each value represents an average of three measurements. PtoS, closed circle; PtoR, open circle; 11-12, closed triangle; 13-8, open triangle.

(C) Growth of T1 and T1(*avrPto*) on Money Maker and 48-2 leaves. Leaf bacteria were measured as given in **(B)**. Money Maker infected with T1, closed circle; 48-2 plant infected with T1, open circle; Money Maker infected with T1(*avrPto)*, closed triangle; 48-2 plant infected with T1(*avrPto*), open triangle.

(D) Growth of T1(*avrPto*) on PtoS and PtoR leaves. Leaf bacteria were measured as given in **(B)**. PtoS infected with T1(*avrPto*), closed circle. PtoR infected with T1(*avrPto*), open circle.

Error bars indicate standard error.

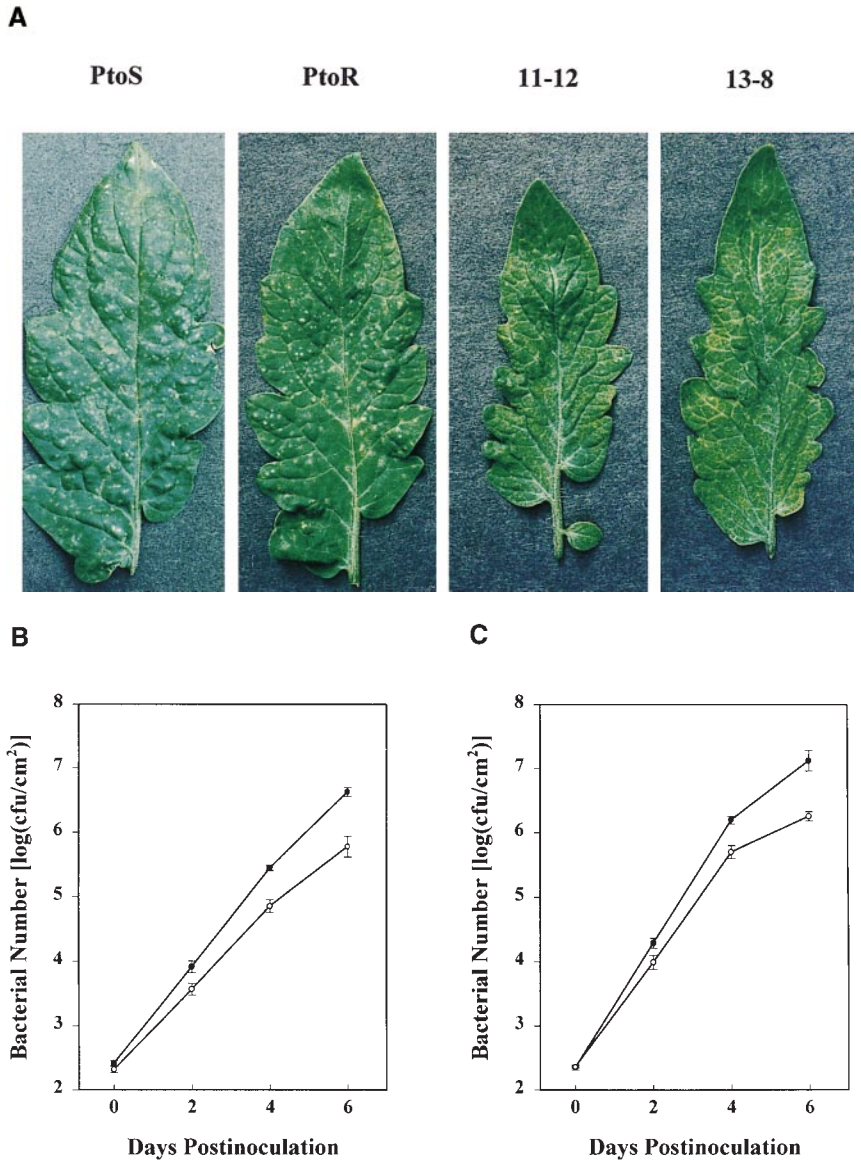

Figure 7. Increased Resistance of 35S::*Pto* Plants to *X. c. vesicatoria.*

Six-week-old tomato plants were vacuum-infiltrated with two strains of *X. c. vesicatoria*, 75-3 and 90-14, at a concentration of 10^4 cfu/mL (in 10 mM $MgCl_2$ plus 0.04% Silwet). Bacterial growth on Money Maker and 48-2 leaves was monitored at 0, 2, 4, and 6 days after inoculation.
(A) Disease symptoms caused by 75-3. Inoculated leaves were photographed 6 days after inoculation.
(B) Growth of 90-14 strain on Money Maker and 48-2 plants. Leaf bacteria were measured as given in Figure 6B. Money Maker, closed circle; 48-2, open circle.
(C) Growth of 75-3 strain on Money Maker and 48-2 plants. Leaf bacteria were measured as given in Figure 6B. Money Maker, closed circle; 48-2, open circle.
Error bars indicate standard error.

DISCUSSION

Pto Overexpression Confers Race-Nonspecific Resistance in Tomato Plants

Pto overexpression in plants constitutively activates defense responses and results in general resistance in the absence of the *avrPto* gene. This is seemingly inconsistent with the previous understanding of *Pto* as a race-specific resistance gene that confers induced resistance when *avrPto* is present in the pathogen. *Pto* is known to encode a protein kinase that specifically interacts with the AvrPto protein, and binding of AvrPto to Pto determines the resistance outcome, presumably by activating the Pto kinase (Scofield et al., 1996; Tang et al., 1996). However, the observed general resistance in plants overexpressing *Pto* may be explained by the biochemical properties of the Pto kinase. It has been demonstrated previously by using an in vitro kinase assay that a recombinant Pto kinase expressed in *Escherichia coli* is capable of autophosphorylation as well as cross-phosphorylation of Pti1, a Pto-interacting protein, in the absence of the AvrPto protein (Loh and Martin, 1995; Zhou et al., 1995). In addition, interactions of Pto with various Pto-interacting (Pti) proteins in the yeast two-hybrid assay are independent of AvrPto but require the kinase activity of Pto (Zhou et al., 1995). Together, these findings suggest that the Pto kinase alone has a basal activity to trigger downstream defense responses.

Indeed, we found that PtoR plants showed marginal but reproducible resistance to the T1 strain of *P. s. tomato.* In PtoR plants, Pto kinase activity is likely to be maintained at a low level because of the low cellular concentration of the Pto protein and the action of counteracting protein phosphatases, as observed in many other signaling systems (Hunter, 1995). However, binding of AvrPto may increase the kinase activity of Pto, presumably by stabilizing or changing the conformation of the Pto kinase. The increased Pto kinase activity enhances the phosphorylation of Pto substrates, thus leading to various resistance responses. It is thus conceivable that an increase in Pto protein abundance could magnify kinase activity and therefore enhance the resistance to a pathogen without requiring the presence of AvrPto. The observed race-nonspecific resistance to the bacterial and fungal pathogens in plants overexpressing *Pto* supports this possibility.

The enhanced race-nonspecific disease resistance triggered by *Pto* overexpression provides a novel strategy to engineer general resistance by using cloned *R* genes. It is particularly attractive because the resulting race-nonspecific resistance is likely to be durable. At this point, we do not know whether the enhanced race-nonspecific resistance of the 35S::*Pto* plants to *P. s. tomato*, *X. c. vesicatoria*, and *C. fulvum* is significantly expressed in the field. Nevertheless, our findings suggest that a cloned *R* gene may be used to enhance resistance to normally unrelated pathogens or pathogen races.

Genes homologous to *Pto* have been found in a number of plant species, and some of them have been mapped to known disease resistance loci (Martin et al., 1993; Botella et al., 1997). However, whether these *Pto* homologs also confer disease resistance function awaits investigation. *Pto* is unique among the cloned *R* genes because it encodes a cytoplasmic protein kinase (Baker et al., 1997). Therefore, it will be of interest to determine how our findings apply to other cloned *R* genes. *Xa21*, an *R* gene cloned from rice, also encodes a protein kinase, but it has an extracellular leucine-rich repeat (LRR) domain and is likely to be a membrane-localized receptor kinase (Song et al., 1995). The Xa21 protein possesses kinase activity in an in vitro kinase assay (P. Ronald, personal communication). However, whether overexpression of *Xa21* activates defense responses in rice plants remains to be determined. All other *R* genes cloned to date encode proteins with an LRR domain, and the biochemical functions of these *R* genes are unclear (Baker et al., 1997). It has been hypothesized that these *R* genes may function in concert with a protein kinase to activate disease resistance (Baker et al., 1997).

Overexpression of LRR-type *R* genes might also confer enhanced race-nonspecific resistance, provided that the abundance of the *R* gene products is rate limiting in the activation of disease resistance. One example of this is provided by the recent observation that general resistance occurs in tomato plants harboring multiple copies of *Prf*, an LRR-type gene that is required for *Pto* function (Oldroyd and

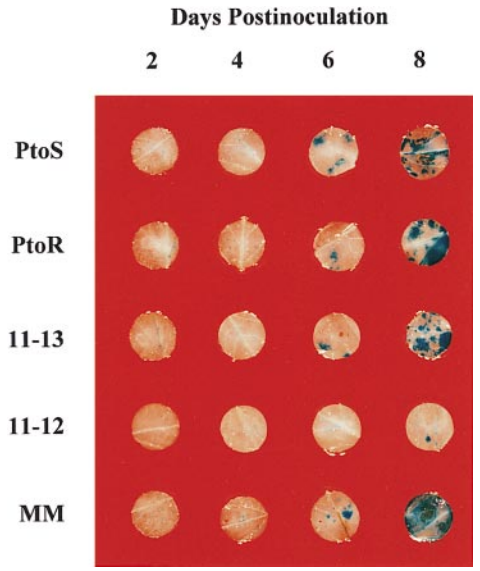

Figure 8. Increased Resistance to *C. fulvum* by *Pto* Overexpression.

Tomato leaves were inoculated with conidia of *C. fulvum.* Leaf discs were removed at the times indicated, and the biomass of *C. fulvum* was measured by staining for GUS activity. Blue stain indicates a high level of *C. fulvum* growth in leaves. 11-13 is a sibling line of 11-12 but does not contain the *Pto* transgene. MM, Money Maker.

Staskawicz, 1998). No spontaneous cell death was reported for the plants overexpressing *Prf.* However, it should be noted that the *Prf* transgene in this report was controlled by the native promoter that resulted only in a small increase in *Prf* transcripts. Whether expression of *Prf* at high levels induces spontaneous cell death remains to be determined.

Pto Overexpression and Cell Death

Mutants with a necrotic lesion phenotype have been documented in many plant species (reviewed in Dangl et al., 1996). Because cell death in many of these lesion mimic mutants is correlated with the expression of histochemical and molecular markers associated with disease resistance, these mutant genes may define important signal transduction components that regulate cell death in the HR (Dietrich et al., 1994; Greenberg et al., 1994). However, ectopically expressed genes with diverse functions from a variety of sources can also cause lesion formation in plants, suggesting that metabolic perturbation may also result in cell death (Becker et al., 1993; Mittler et al., 1995; Dangl et al., 1996; Herbers et al., 1996; Abad et al., 1997). The observed cell death in plants overexpressing *Pto* provides direct evidence that misregulation of *R* genes could lead to spontaneous cell death.

Similar to some lesion mimic mutants, induction of cell death in plants overexpressing *Pto* requires intense light, indicating that accumulation of the Pto protein itself is insufficient to activate the cell death pathway. It is known that the generation of reactive oxygen species (ROS) is required for the HR as well as for lesion formation in the Arabidopsis *lsd1* mutant (Low and Merida, 1995; Jabs et al., 1996). The requirement of light for cell death induction in 35S::*Pto* plants suggests that ROS generated during photosynthesis interact with the Pto kinase, either directly or indirectly, to induce cell death in plants overexpressing *Pto.* This is consistent with the localized cell death in the palisade cell, where photosynthesis is most active. Because ROS may play a direct role in cell suicide (Levine et al., 1994), two possibilities exist for the observed interplay between light and *Pto* overexpression. First, overexpression of *Pto* may result in an elevated but sublethal ROS concentration. The lethal concentration is reached when additional ROS are generated under strong light during photosynthesis in the palisade cell. Similar examples can be found in the light-inducible cell death in plants expressing the antisense RNA of a catalase gene (Takahashi et al., 1997; Chamnongpol et al., 1998) and transgenic plants expressing a glucose oxidase gene (Kazan et al., 1998). The second possibility is that the activation of the Pto pathway by *Pto* overexpression may inhibit H_2O_2 scavenging enzymes and therefore potentiate the sensitivity of leaves to ROS produced during photosynthesis. For example, increased levels of SA could directly inhibit the activity of ascorbate peroxidase and catalase, two H_2O_2 scavenging enzymes (Chen et al., 1993; Durner and Klessig, 1995). Mittler et al. (1998) recently reported that inoculation of tobacco mosaic virus and *P. s.* pv *phaseolicola* on tobacco leaves induces the HR and reduces ascorbate peroxidase protein concentration in the plant.

Activation of Defense by *Pto* Overexpression

In addition to spontaneous cell death, *Pto* overexpression also led to callose deposition and lignification, accumulation of SA, constitutive induction of acidic and basic *PR* genes, and enhanced resistance to virulent pathogens. These are all indicative of constitutive defense activation in plants overexpressing *Pto.* It has been demonstrated in a number of systems that SA is required not only for SAR but also for local resistance (Ryals et al., 1996). Inoculation of PtoR plants with DC3000, an avirulent strain of *P. s. tomato*, also results in SA accumulation (Oldroyd and Staskawicz, 1998), although it is not known whether this is sufficient to protect the tomato plant from a second infection by pathogens.

The accumulation of SA in the 35S::*Pto* plants may play a crucial role in the observed disease resistance. However, this does not preclude SA-independent defense responses being part of the observed resistance. In fact, SA-independent SAR mechanisms have been described for Arabidopsis (Pieterse et al., 1996; Bowling et al., 1997). We have shown previously that Pto interacts with another protein kinase, Pti1, and the transcription factors Pti4, Pti5, and Pti6 (Pti4/5/6; Zhou et al., 1995, 1997). Pti1 is involved in the HR, whereas Pti4/5/6 are believed to regulate basic *PR* gene expression in response to pathogens (Zhou et al., 1995, 1997). The overexpression of *Pto* may directly activate multiple defense pathways through the downstream components, such as Pti1, Pti4, Pti5, and Pti6. The constitutive activation of *Pti5* expression in plants overexpressing *Pto* supports this possibility. It will be interesting to determine whether cell death in plants overexpressing *Pto* can be uncoupled from defense gene expression and general resistance. In the Arabidopsis mutant *dnd1*, disease resistance occurs in the absence of the HR during gene-for-gene interaction (Yu et al., 1998), suggesting that the HR and other defense responses are separable.

In summary, we describe the constitutive activation of cell death and general defense responses in tomato plants overexpressing the *Pto* gene. The race-nonspecific resistance in these plants demonstrates that it might be possible to engineer for durable and broad-spectrum resistance by overexpressing cloned *R* genes.

METHODS

Plant Materials

Money Maker, Rio Grande–PtoS, and Rio Grande–PtoR are the tomato (*Lycopersicon esculentum*) cultivars that were used. The

35S::*Pto* transgene was introduced into Money Maker plants, as described previously (Martin et al., 1993; Loh et al., 1998). Two original transgenic plants (numbers 11 and 13) were preserved by backcrossing to Rio Grande–PtoS plants (Martin et al., 1993). 11-12 and 13-8 were derived from the backcrossed progenies and contained the *Pto* transgene. 11-13 is a sibling of 11-12 from the backcrossed progeny that does not carry the *Pto* transgene. 48-2 is a selfed line from a 35S::*Pto* transgenic Money Maker plant carrying a single copy of the *Pto* transgene tagged with a hemagglutinin epitope (Loh et al., 1998). Tomato seeds were germinated in autoclaved Bacto potting soil (Swecker-Knipp, Topeka, KS). Plants were grown in a greenhouse at a constant temperature of 28°C. Healthy and well-expanded leaves from 6-week-old plants were used for gene expression and the disease resistance assays.

RNA Extraction and RNA Gel Blot Analysis

Well-expanded leaves from 6-week-old plants were collected for gene expression analysis. Leaves were immediately frozen in liquid nitrogen after collection, and RNA was isolated as previously described (Tang et al., 1994). Ten micrograms of total RNA from each sample was loaded on a formaldehyde–agarose gel, separated by electrophoresis, transferred to a nitrocellulose membrane, and immobilized to the nitrocellulose membrane by UV cross-linking. cDNA probes for the *Pto*, *Pti4*, *Pti5*, *Chia*, *Glua*, and *Glub* genes were as previously described (van Kan et al., 1992; Danhash et al., 1993; Martin et al., 1993; Zhou et al., 1997). *PR-1a1* and *PR-1b1* cDNAs were amplified by polymerase chain reaction by using specific primers based on published sequences (Tornero et al., 1994, 1997) and are as follows: 5′-TAGGATCCTCACTCATCACAAGCTC-3′ and 5′-AAGAATTCCACTCATACATGACTGATG-3′ for *PR-1a1* and 5′-AAGGATCCGACCATTTTTATCATTTCCTCT-3′and 5′-ATGGAATTCTTGTATGAGAGAATATGGAAGACTTG-3′ for *PR-1b1.*

All probes were radiolabeled with ^{32}P-dCTP by using a random-primed labeling kit (Amersham Corp.). RNA gel blot analysis was performed as previously described (Tang et al., 1994). Briefly, the nitrocellulose membrane was incubated in a buffer containing 50% formamide, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 200 μg/mL heterologous DNA, 0.5% SDS, and 2% dextran sulfate for 2 hr at 42°C before hybridization. Hybridization was performed in the same buffer containing denatured cDNA probe for 16 hr at 42°C. The membrane was washed three times (15 min each time) in 2 × SSPE plus 0.1% SDS at 42°C, and hybridization was visualized by autoradiography.

Histochemistry and Microscopy

Cells undergoing cell death in tomato leaves were photographed by using a dissecting microscope. Methods described by Dietrich et al. (1994) were used for the detection of autofluorescent materials and callose deposition. Briefly, leaves were cleared by boiling in lactophenol and rinsed first in 50% ethanol and then in water. The cleared leaves were examined with a light microscope for the presence of nontransparent cells. To visualize autofluorescence, we examined cleared leaves with a UV epifluorescence microscope. For analysis of callose deposition, we stained cleared leaves for 1 hr at room temperature in a solution containing 0.01% (w/v) of aniline blue and 0.15 M K_2HPO_4 and examined them using the epifluorescence microscope. For trypan blue staining, we boiled leaves in lactophenol containing 0.01% trypan blue for 10 min and rinsed them in 50% ethanol and then in water. Stained leaves were examined with a light microscope.

Leaf material for sectioning was fixed in FAE (3.7% formaldehyde, 5% acetic acid, and 45% ethanol), dehydrated, and embedded in paraffin. Embedded leaves were sectioned on a microtome at a thickness of 10 μm. Leaf sections were mounted on microscope slides, dewaxed, and rehydrated. For detection of autofluorescence, sections were dehydrated in an ethanol series, cleared in xylene, mounted on a slide with a cover slip and mounting medium, and examined using a UV epifluorescence microscope. To stain for lignin, the rehydrated tissues were stained in 2% $FeNH_4SO_4$ for 20 min and then in 0.5% hematoxylin. Sections were then dehydrated in an ethanol series and stained first with safranin (1:25,000 in 50% ethanol) for 30 min and then with Fast Green (Sigma) (1:25,000 in 3:1 xylene:ethanol) for 5 min during the dehydration process. The sections were cleared in xylene, mounted on a slide, and examined with a light microscope. Images were obtained using a CCD (charge-coupled device) camera coupled to the microscope, and the digital image was implemented using a Macintosh computer. Photographs were pseudocolored using the software Adobe Photoshop 5.0 and printed with a color printer.

Measurement of Salicylic Acid

Salicylic acid (SA) and SA glucoside (SAG) were measured by using leaves from 6-week-old plants. SA and SAG were extracted and analyzed as previously described (Bowling et al., 1994).

Pathogen Infections of Tomato Leaves

The T1 strain of *Pseudomonas syringae* pv *tomato* and strains 75-3 and 90-14 of *Xanthomonas campestris* pv *vesicatoria* were grown on King's B (KB) plates (Martin et al., 1993) containing 50 mg/L rifampicin. The *P. s. tomato* T1(*avrPto*) strain was grown on a KB plate containing 50 mg/L rifampicin and 50 mg/L kanamycin. Bacteria were grown overnight in liquid KB medium with the appropriate antibiotics. The bacterial culture was washed twice with 10 mM $MgCl_2$ and resuspended in 10 mM $MgCl_2$. Bacterial density was determined by absorbence at $OD_{600\ nm}$. Bacteria were diluted to the desired concentrations in 10 mM $MgCl_2$ plus 0.04% Silwet L-77 (Osi, Danbury, CT) for inoculation. Six-week-old plants were inoculated by vacuum infiltration, and the inoculated plants were kept in a greenhouse. Leaf bacteria were measured by grinding two leaf discs in 10 mM $MgCl_2$, plating diluted suspensions on appropriate KB plates, and counting colony-forming units (cfu).

A race 4 strain of *Cladosporium fulvum* expressing the β-glucuronidase (*GUS*) gene (Oliver et al., 1993) was kindly supplied by J.D.G. Jones (Sainsbury Laboratory, John Innes Centre, Norwich, UK). The strain was subcultured on quarter-strength potato–dextrose agar at 23°C. Conidia from 3-week-old cultures on potato–dextrose agar were used for inoculation. The third or fourth leaf of 4-week-old tomato plants was inoculated on the abaxial side by spraying with a suspension of 10^5 conidia per mL. Plants were allowed to dry for 1 hr and then sprayed twice again to provide a dense and homogeneous inoculum. After spraying, plants were put in a polyethylene-covered box and incubated in a growth chamber for 3

days in the dark on trays partially filled with water to maintain a relative humidity of 100%. On the third day after spraying, we opened the box to promote stomatal opening. We used the procedure described by Jefferson et al. (1987) to examine the GUS activity of *C. fulvum* in the infected leaves. Briefly, 10 leaf discs (1 cm in diameter) from each tomato plant were cut with a cork borer and vacuum infiltrated under reduced pressure for 2 to 5 min with a solution containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, sodium salt (U.S. Biological, Swampscott, MA), 0.05% (v/v) Triton X-100, 1 mM potassium ferricyanide/ferrocyanide, 1 mM EDTA, and 50 mM phosphate buffer, pH 7, and incubated in the dark at 37°C for 16 hr. After staining, we removed chlorophyll from the stained samples by incubation in 70% ethanol at room temperature for several days. Photographs were taken under tungsten light with Kodak Ektachrome film.

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